INSECT TOLERANT TRANSGENIC TOMATO PLANTS

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The structure of an insect control protein gene from *Bacillus thuringiensis* var. *kurstaki* HD-1 was determined, and truncated forms of the gene that express a functional insecticidal protein were generated. Two of these truncated genes were incorporated into a plant expression vector for *Agrobacterium*-mediated transformation. Transgenic tomato plants containing the chimeric genes express the insect control protein gene. Such expression confers tolerance to lepidopteran larvae on the transgenic tomato plants and their progeny. These engineered tomato plants represent a significant step to increased selectivity, specificity and efficiency in insect control.

Bacillus thuringiensis (B.t.) is an entomocidal, spore-forming bacterium. Most strains of *B.t.* are specifically lethal to lepidopteran insects (e.g., larvae of moths and butterflies)[1]. The insecticidal activity of *B.t.* resides in a parasporal protein crystal that is formed upon sporulation[2]. Crystal/spore preparations of *B.t.* have been used as commercial insecticides in products such as Dipel® (Abbott Laboratories) for many years. The commercial *B.t.* insecticides are effective against more than 50 lepidopteran pest species[3]. In the lepidopteran-type *B.t.* strains, the crystal is composed of insect control protein (*B.t.* protein) subunits of approximately 130 kD[4]. Several genes encoding lepidopteran-type *B.t.* proteins have been isolated and their DNA sequences determined[5–11].

In the past few years, it has become possible to introduce and express foreign genes in plant cells, especially through the use of *Agrobacterium*-mediated transformation[12]. For many species, transgenic plants containing and expressing such foreign genes have been regenerated. This technology has recently been applied to the genetic engineering into transgenic plants of agronomically important traits such as tolerance to the herbicide Roundup®[13,14] and tolerance to plant viruses[15].

Chemical control of insect pests is estimated to cost more than $3 billion per year worldwide. Over $400 million is spent each year for control of lepidopteran pests in the U.S. alone. The genetic engineering of insect tolerance into crop plants is therefore a goal of significant interest to agricultural biotechnology. A logical approach to this problem is through the expression of insect control protein genes from *B.t.* (*B.t.* genes) in transgenic plants. In this report we describe the construction of chimeric genes containing truncated forms of a lepidopteran-type *B.t.* gene that have been engineered for expression in plants. These genes have been introduced into tomato cells and transgenic plants have been recovered. We demonstrate that the chimeric *B.t.* genes are expressed in tomato plants and that expression of the insect control protein gene confers insect tolerance on the transgenic plants and their progeny.

RESULTS

Sequence of a *B.t.* insect control protein gene. We have previously described the isolation and initial subcloning of a lepidopteran-type insect control protein gene from *B.t.* var. *kurstaki* HD-1 (*B.t.k.* gene)[16]. This gene was contained on a BamHI-PstI fragment of approximately 4.5 kilobase pairs (kb) in plasmid pMAP4 (Fig. 2). We have determined the nucleotide sequence of 3734 base pairs (bp) of *B.t.k.* DNA contained in pMAP4 including the entire protein encoding segment. As shown in Figure 1 the sequence determined contains an open reading frame of 3468 bp encoding a protein of 1156 amino acids with a predicted molecular weight of 130,690. This is in good agreement with the size of the protein estimated by polyacrylamide gel electrophoresis. As previously described, the *B.t.k.* gene contained in pMAP4 expresses an insect control protein (*B.t.k.* protein) that is lethal to lepidopteran larvae when expressed either in *E. coli* or in *Pseudomonas fluorescens*[16,17].

Several groups have reported nucleotide sequences for lepidopteran-type *B.t.* genes[5–11]. We have compared both the nucleotide sequence and the derived amino acid sequence of the insect control protein gene shown in Figure 1 with these other reported sequences. Based on this analysis, the gene described here is nearly identical to a recently reported gene also isolated from *B.t.k.* HD-1 and designated *kurhd1*[11]. Our *B.t.k.* gene and *kurhd1* differ by just nine nucleotides in the coding region. These nucleotide differences account for four amino acid differences between the proteins including a one amino acid deletion in *kurhd1* relative to our gene. Two groups have reported nucleotide sequences for an insect control protein gene from *B.t.*. var. *berliner* 1715[9,10]; these two reported *berliner* sequences differ from one another by only two amino acid changes. Interestingly, our *B.t.k.* gene encodes a protein nearly identical to that encoded by the *berliner* genes; it differs from the gene of Wabiko et al.[9] by four amino acids and from the gene of Hofte et al.[10] by six amino acids.

Others have reported detailed comparisons between the published lepidopteran-type *B.t.* gene sequences[9–11]. We

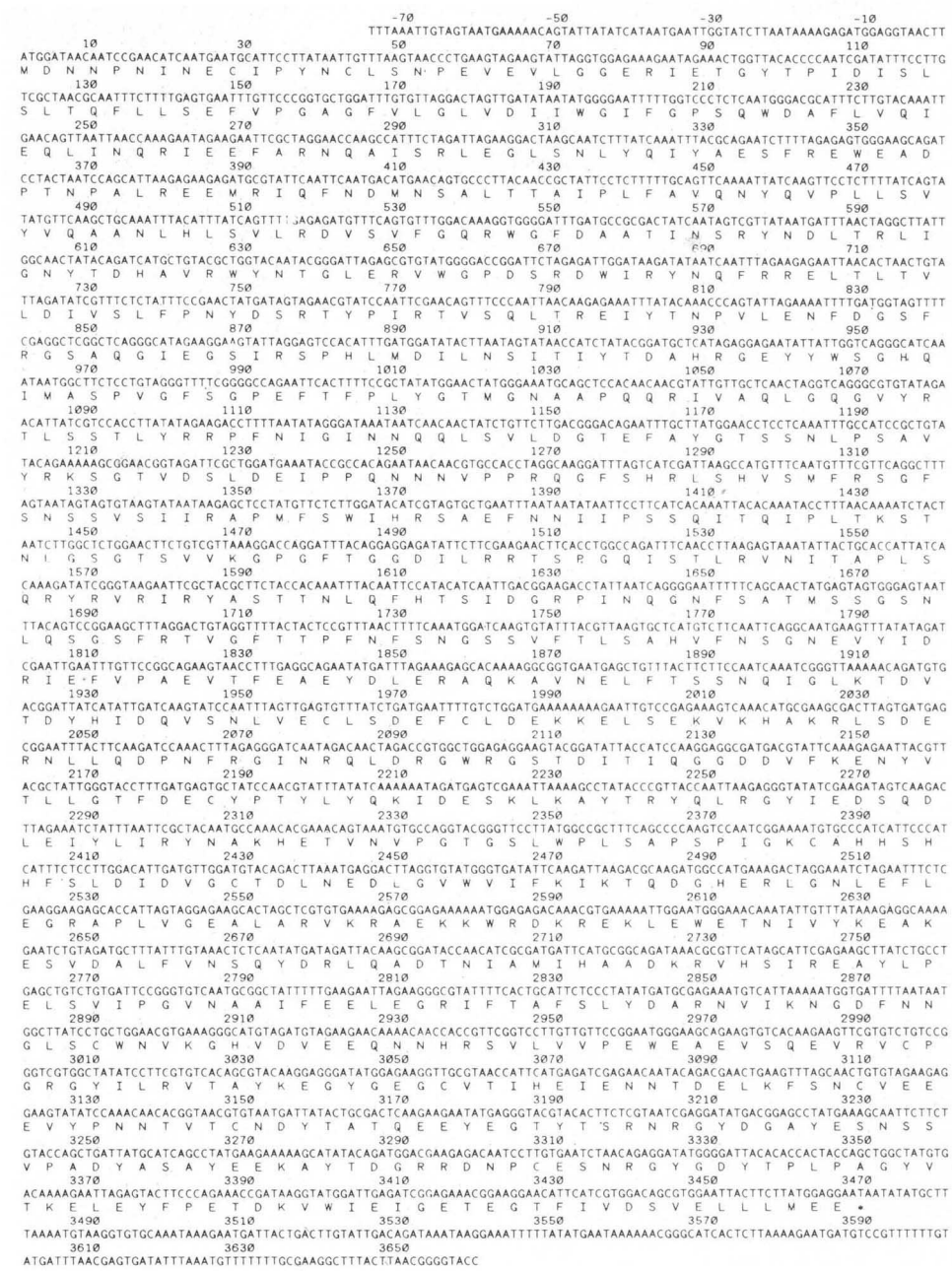

FIGURE 1 DNA sequence and derived amino acid sequence of the insect control protein gene from *B.t.* var. *kurstaki* HD-1. The sequence shown includes the entire *B.t.k.* protein coding sequence, 75 nucleotides of 5′ flanking sequence and 188 nucleotides of 3′ flanking sequence. Nucleotide coordinates are numbered from the first nucleotide of the translational initiation codon. The derived amino acid sequence is shown in the one letter amino acid code.

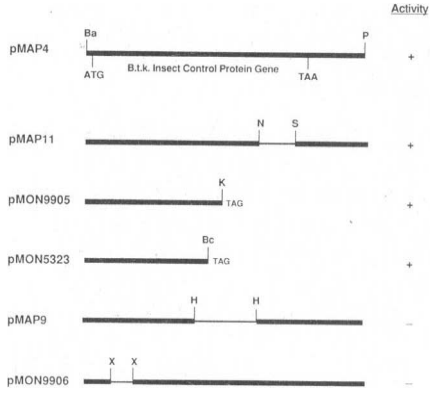

FIGURE 2 Deletion analysis of the *B.t.k.* insect control protein gene. The top line shows the structure of the 4.5 kb *B.t.k.* gene fragment in pMAP4. The initiation and termination codons for the protein are indicated. The deletion variants described in the text are shown below. The narrow lines indicate regions of the gene that have been deleted. Restriction enzyme cleavage sites are indicated as Ba, BamHI; Bc, BclI; H, HindIII; K, KpnI; N, NruI; S, ScaI; X, XbaI. The nucleotide coordinates of the indicated restriction sites are given in the text. pMON9905 and pMON5323 contain truncated *B.t.k.* genes fused to termination codons (indicated as TAG) provided by synthetic oligonucleotide linkers. The column on the right summarizes data on lethality to *M. sexta* larvae of the truncated *B.t.k.* proteins encoded by the variants.

have compared, in pairwise combinations, all of the published sequences, and our results confirm and extend these earlier reports (data not shown). In summary, the lepidopteran-type insect control proteins are highly homologous. However, they include a highly variable region that can extend from approximately amino acid 285 through amino acid 640. Based on differences in this variable region, the genes reported to date can be assigned to four classes. One class includes the *B.t.k.* gene described here, the *kurhd1* gene and the two *berliner* genes. A second class includes a gene from *B.t.* var. *sotto*[6] and the gene from *B.t.k.* HD-1 described by Schnepf et al.[5]. A gene from *B.t.k.* HD-73[7] forms the third class, and the fourth class contains another gene from *B.t.k.* HD-1 described by Thorne et al.[8].

Deletion analysis of the *B.t.k.* gene. It is known that proteolytic fragments of *B.t.* proteins, which are only about half the size of the intact protein, are active against insects[18,19]. In order to define shorter segments of the *B.t.k.* gene, which would still encode this functionally active domain, we have created a variety of deletion variants. These variants have been expressed in *E. coli*, and in all cases accumulation of protein of the expected size was observed by Western blot analysis utilizing antibodies prepared against purified crystal protein (data not shown). *E. coli* cultures expressing the deletion variants have been assayed for insect control activity by bioassay with neonate larvae of *Manduca sexta* (tobacco hornworm). The deletion variants analyzed and the results of the bioassay are shown in Figure 2.

The *B.t.k.* gene in pMAP11 contains a deletion extending from a NruI site at nucleotide 2704 to a ScaI site at nucleotide 3376 (nucleotide coordinates refer to the numbering in Fig. 1). This deletion causes an in-frame fusion and encodes an insect control protein lacking amino acids 902 to 1126. When expressed in *E. coli* this protein was lethal to *M. sexta*. pMON9905 contains a *B.t.k.* gene truncated at the KpnI site at nucleotide 2174. A synthetic oligonucleotide linker with two in-frame termination codons has been inserted in the KpnI site. The protein produced by this gene contains the first 725 amino acids of the intact insect control protein, and it was lethal to *M. sexta*. The *B.t.k.* gene in pMON5323 is truncated at the BclI site at nucleotide 1935 and also has a termination codon linker attached at this site. This truncated gene encodes a protein containing the first 646 amino acids of the intact insect control protein plus a four amino acid C-terminal extension encoded by the linker. This protein was also lethal to *M. sexta*. pMAP9 was created by deletion of the HindIII fragment extending from nucleotide 1692 to nucleotide 2747. The gene created by this manipulation encodes the first 565 amino acids of the intact insect control protein plus a three amino acid C-terminal extension created by the deletion. This truncated *B.t.k.* protein had no activity against *M. sexta*. One additional variant (pMON9906) was created by deletion of the XbaI fragment extending from nucleotide 292 to nucleotide 667. This deletion causes an in-frame fusion and encodes a protein lacking amino acids 98 through 222. This protein was not active in the bioassay.

Based on the analysis described above, the N-terminal half of the *B.t.k.* protein is essential for lethal activity, but the C-terminal half is dispensable. A truncated protein containing just the first 646 amino acids was a functional insect control protein. However, a protein containing the first 565 amino acids was not active. In addition, a deletion of 125 amino acids in the N-terminal portion of the protein (pMON9906) was sufficient to eliminate lethal activity. These results are consistent with the results obtained from similar deletion analysis performed on other lepidopteran-type *B.t.* genes[6,7,9,10,20].

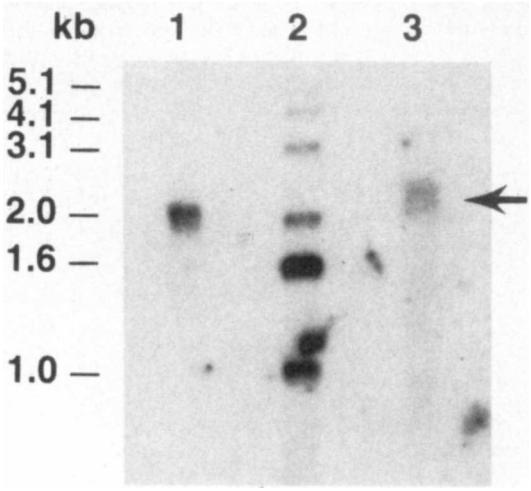

FIGURE 4 Northern hybridization analysis of *B.t.k.* mRNA in a pMON9711 transgenic plant. RNA was isolated from callus derived from leaves of pooled kanamycin resistant R1 progeny of plant #337, a pMON9711 transformant. The RNA was fractionated on a formaldehyde-containing agarose gel and blotted to a GeneScreen membrane. The membrane was hybridized with ^{32}P-labeled 4.5 kb BamHI-PstI fragment from pMAP17 containing the entire *B.t.k.* gene. Lane 1, *B.t.k.* gene DNA marker, the 2.2 kb BglII fragment from pMON9711 (2 ng). Lane 2, ^{32}P-labeled 1 kb ladder (Bethesda Research Laboratories). Sizes of the ladder fragments are indicated on the left. Lane 3, Poly A$^+$ RNA (25 μg) isolated from callus as described above. The arrow to the right indicates the position of the hybridizing *B.t.k.* RNA in lane 3.

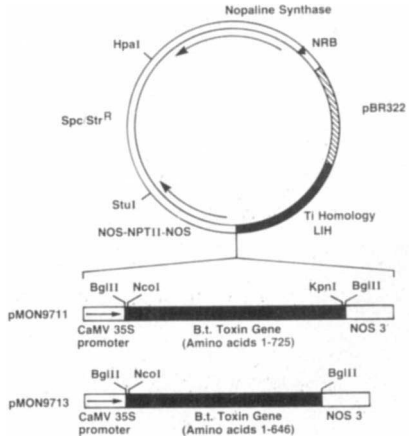

FIGURE 3 Plant transformation vectors containing chimeric truncated *B.t.k.* insect control protein genes. The structure of the chimeric *B.t.k.* gene contained in each vector is indicated. The CaMV 35S promoter is derived from cauliflower mosaic virus. Polyadenylation signals are derived from the nopaline synthase gene (NOS 3′). Important functional regions of the plant transformation vector are shown in the circular diagram above. NOS-NPTII-NOS, a chimeric gene conferring kanamycin resistance on plant cells; Spc/Str^R, a bacterial spectinomycin/streptomycin resistance gene; pBR322, the origin of replication from pBR322; the nopaline syntase gene from the Ti plasmid; NRB, the right border of the T-DNA from a nopaline Ti plasmid; LIH, a segment of the octopine Ti plasmid that provides a region of homology for cointegrate formation with disarmed Ti plasmids.

Plant transformation vectors containing truncated *B.t.k.* genes. Chimeric *B.t.k.* genes containing the CaMV 35S promoter, and either the full length *B.t.k.* protein coding sequence or the coding sequence for active truncated variants as defined by the deletion analysis, were constructed for expression in plants. The frequency at

which we were able to recover transgenic plants that exhibited detectable expression (by bioassay) of the chimeric *B.t.k.* gene was significantly greater when truncated genes were employed than when the full length gene was used. Initially, two transgenic tomato plants transformed with pMON9711 (which contains a truncated *B.t.k.* gene, Fig. 3) were recovered. When assayed for insect control activity, both of these plants displayed a high level of activity against *M. sexta* larvae (100% mortality in repeated bioassays). At the same time we recovered and assayed three transgenic plants containing a full length chimeric *B.t.k.* gene. Only one of these three plants had detectable activity, and the level of activity (50% to 80% mortality in repeated bioassays) was lower than for the plants containing the truncated gene. We thus focused our attention on the transgenic plants containing the truncated chimeric genes, and these results are described below.

Chimeric truncated *B.t.k.* genes were constructed for expression in plants as follows. The active truncated deletion variants contained in pMON9905 and pMON5323 were further modified by addition at their 5′ ends of a linker containing a BglII site (see Experimental Protocol). These truncated insect control protein genes were moved as BglII fragments into the BglII site of pMON316, a plant expression cassette vector[21]. pMON316 is a plant transformation vector based on pMON200; it contains an expression cassette consisting of the 35S promoter of cauliflower mosaic virus (CaMV) and the 3′ end of the nopaline synthase (NOS) gene from the Ti plasmid of *Agrobacterium* with a BglII site between these two elements. The resulting plant transformation vectors, pMON9711 and pMON9713, containing truncated chimeric *B.t.k.* genes are shown in Figure 3. These vectors were integrated into the disarmed Ti plasmid of *Agrobacterium tumefaciens* for transformation of plants[22].

Expression of *B.t.k.* genes in tomato plants. *Agrobacterium* strains containing pMON9711 and pMON9713 were used to transform tomato explants[23], and transgenic tomato plants containing the *B.t.k.* genes were recovered at a frequency of approximately three kanamycin resistant plants per one hundred explants inoculated with *Agrobacterium*. Expression of the *B.t.k.* gene contained in pMON9711 was assayed in one such plant by Northern hybridization analysis. As shown in Figure 4 (lane 3) this plant produced a polyadenylated mRNA that hybridized to a *B.t.k.* gene probe. The size of the mRNA estimated from this gel was approximately 2500 nucleotides. This is the expected size of a full length *B.t.k.* mRNA from pMON9711 given the size of the *B.t.k.* protein encoding BglII fragment and the known sites for transcriptional initiation from the CaMV35S promoter[24] and for polyadenylation by the NOS 3′ end[25]. For the experiment shown in Figure 4, RNA was isolated from callus derived from leaves of pooled kanamycin resistant R1 progeny of a transgenic plant (#337). We have detected a similar full length *B.t.k.* mRNA in leaves of this plant and an additional pMON9711 transformant; however, for R1 progeny of plant #337 the *B.t.k.* mRNA appeared to be more abundant in callus than in leaves. The hybridization analysis indicated that the expression level of the *B.t.k.* gene was quite low. The level of *B.t.k.* mRNA was substantially lower than the level of NPTII mRNA in these plants even though the NPTII gene is expressed from the nopaline synthase (NOS) promoter (Fig. 3) which is reported to be approximately 30-fold less active than the CaMV 35S promoter[21] driving the *B.t.k.* gene. In spite of the low abundance of *B.t.k.* mRNA, plants expressing the gene at this level are lethal to insects as described below.

Insect control activity of transgenic tomato plants against lepidopteran larvae. Tomato plants containing pMON9711 and pMON9713 have been tested for lethality to lepidopteran larvae utilizing either isolated leaves or whole plants in feeding assays. Figure 5 shows an example of the isolated leaf bioassay against larvae of *M. sexta*. As can be seen in the figure, after four days of feeding ten neonate larvae were able to consume an entire leaf from a nontransformed plant. There was no mortality, and the larvae increased in size approximately five-fold. In contrast, the larvae feeding on the pMON9711 transgenic plant were all killed within 48 hours, and there was very little evidence of feeding damage to the leaf. This lack of damage by larvae feeding on the transgenic plant is consistent with the known effects of *B.t.* insect control protein. One early sign of *B.t.* intoxication is an inhibition of feeding by larvae[26]. Similar lethality is seen at the whole plant level as illustrated in Figure 6. After seven days of feeding, ten *M. sexta* larvae were able to defoliate completely a six week old nontransformed tomato plant, and the larvae had begun to attack the stems as well as the leaves. Again there was no mortality. The larvae feeding on the pMON9711 transgenic plant were all killed within 72 hours and the plant showed very little evidence of feeding damage.

These results are not limited to the plants shown in the figures. We have recovered ten pMON9711 transgenic plants and five pMON9713 transgenic plants that show similar lethality to *M. sexta*. In the case of pMON9711, 17 kanamycin resistant transgenic plants have been assayed for insect control activity with the *M. sexta* bioassay. Ten of these plants were highly lethal to the larvae (90% to 100% mortality); two plants showed lower, but detectable, activity (20% to 50% mortality), and five had no detectable activity. For pMON9713, four of the five transgenic plants recovered had high activity. We have also tested the insect control activity of these transgenic plants with larvae of two other lepidopterans, *Heliothis zea* (corn earworm) and *Heliothis virescens* (tobacco budworm). Our studies with *E. coli* cultures expressing the cloned *B.t.k.* gene indicate that *M. sexta*, *H. virescens*, and *H. zea*, in that order, are increasingly difficult to kill (data not shown). The results of several typical plant assays are summarized in Table 1. Some of the transgenic plants were able to kill 100% of the *H. virescens* larvae tested, while other plants were not quite as effective. For most of the plants tested, the *H. virescens* larvae that were not killed were severely stunted compared to larvae feeding on control tissue. Typically, larvae on control leaves doubled in size during the four day assay period while larvae on the transgenic leaves showed no weight gain. To date we have tested only one plant against *H. zea*. The results indicate that, as expected, the mortality seen with this insect was somewhat less than that seen with *H. virescens;* however, severe stunting was again seen in all of the surviving larvae. No mortality has been seen in larvae feeding on control transgenic tomato plants transformed with vectors which do not contain a chimeric *B.t.k.* gene (see Table 1).

As is also indicated in Table 1, we have isolated first generation and second generation progeny of these transgenic plants including some putative homozygous progeny. The *B.t.k.* gene segregated with nopaline production and kanamycin resistance as expected. In all of the plants tested to date the *B.t.k.* gene segregated as a single dominant Mendelian marker indicating the presence of a single functional T-DNA locus. As shown in the table, the progeny plants are at least as lethal as the primary transformants, and sometimes display even greater lethality. The results presented here indicate that transgenic tomato plants containing either of the truncated chimeric *B.t.k.* genes are able to express the insect control protein at levels sufficient to kill lepidopteran larvae.

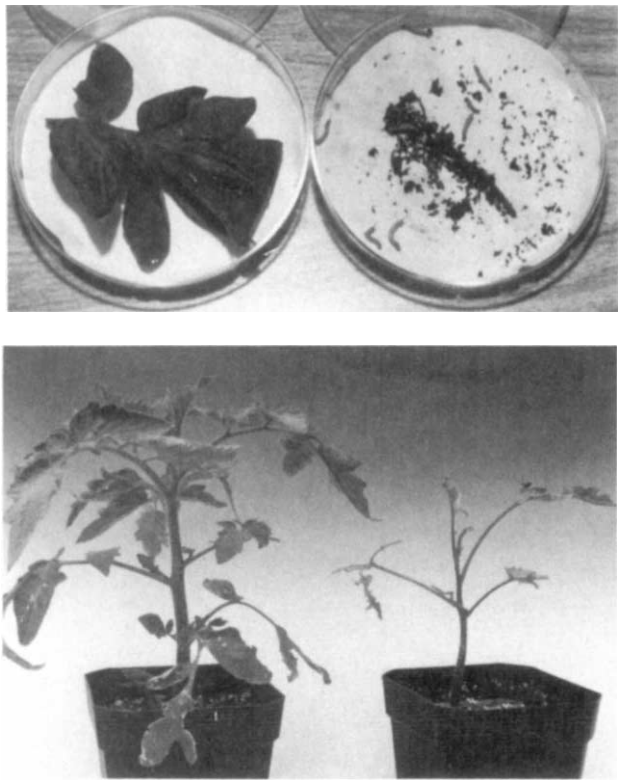

FIGURE 5 Insect bioassay of isolated leaves of a pMON9711 transgenic tomato plant. Isolated leaves were assayed for insect control activity against *Manduca sexta* larvae. Ten neonate larvae were applied to leaves and allowed to feed for four days. Left, leaves from a pMON9711 transgenic tomato plant (#337); dead larvae are visible on the leaf. Right, leaves from a nontransformed control plant; viable larvae are visible.

FIGURE 6 Whole plant insect bioassay of a transgenic tomato plant. Intact tomato plants were assayed for insect control activity against *Manduca sexta* larvae. Ten neonate larvae were allowed to feed on each plant for seven days. Left, a pMON9711 transgenic tomato plant. Right, a nontransformed control plant; viable larvae are visible.

TABLE 1 Toxicity of transgenic tomato plants to lepidopteran larvae.

Plant	Vector (pMON)	Corrected Mortality (%)		
		M. sexta	*H. virescens*	*H. zea*
337 (R0, R2)	9711	100	100	n.d.
344 (R0)	9711	100	2	n.d.
344 (R2)	9711	100	50–70*	10–50*
482 (R1)	9713	100	35–70*	n.d.
306 (R0, R2)	319	0	0	0
379 (R0)	200	0	n.d.	n.d.
Control	none	0	0	0

* Surviving larvae show severe stunting

Results of insect bioassays of pMON9711 and pMON9713 transgenic tomato plants with larvae of *Manduca sexta, Heliothis virescens* and *Heliothis zea*. Bioassays were performed as described in the Experimental Protocol. Results are also shown for control transgenic tomato plants transformed with pMON200[22] or pMON319[15] that do not contain chimeric *B.t.k.* genes and for a nontransformed plant. R0, primary transformed plants regenerated from tissue culture. R1, kanamycin resistant, first generation progeny from selfed R0 plants; a mixture of homozygous and heterozygous transgenic plants. R2, homozygous second generation kanamycin resistant progeny derived from selfed R1 plants.
n.d., not done.

DISCUSSION

In this report we show that expression of a lepidopteran specific insect control protein gene from *Bacillus thuringiensis* confers insect tolerance on transgenic tomato plants. Recently, others have reported that expression of similar *B.t.* insect control protein genes can confer insect tolerance on transgenic tobacco plants (M. Vaeck, personal communication; M. Adang, personal communication). In the experiments reported here we have utilized truncated forms of the gene encoding functional proteins. These genes were expressed from the CaMV 35S promoter, which is known to be a strong promoter in transgenic plants[21]. The chimeric truncated *B.t.k.* gene in pMON9711 was expressed as a full length polyadenylated mRNA. However, the level of *B.t.k.* mRNA was much lower than expected for a chimeric gene expressed from the CaMV 35S promoter. At this point it is not clear why the *B.t.k.* mRNA is not more abundant, but the low level of stable *B.t.k.* mRNA accumulation might indicate that the chimeric transcript is unusually unstable in plant cells. In spite of the low level of RNA expression, the insecticidal protein was expressed at a level sufficient to kill larvae of three important lepidopteran pests of tomato, namely, *M. sexta, H. virescens,* and *H. zea*[27]. The fact that insect control activity is seen even under conditions of low level gene expression is a reflection of the high potency of the *B.t.k.* protein. In the case of the *Heliothis* larvae, although some of the larvae survived four days of feeding on some of the plants tested, the growth of the surviving larvae was severely inhibited. The plants sustained little damage because larval feeding was significantly reduced. Therefore, expression of the protein at levels comparable to that found in the plants described here might provide agronomically significant control of insect pests. These effects, feeding inhibition and stunting of growth at sublethal doses, are known properties of the *B.t.k.* protein[26].

In addition to damaging the foliage of tomato plants, lepidopteran larvae, especially of the *Heliothis* species, can do considerable damage to tomato fruit, making the fruit unmarketable[27]. Newly hatched larvae typically feed on the foliage before feeding on the fruit, so *B.t.k.* expression in leaves might be sufficient to reduce or eliminate fruit damage. For complete protection it might be necessary to have the insect control protein expressed in the fruit as well. We have obtained preliminary evidence that some *B.t.k.* activity is detectable in tomato fruit from our transgenic plants. *Heliothis virescens* larvae fed on transgenic fruit gained weight at only one half the rate of larvae fed on fruit of a nontransformed plant, consistent with a low level of *B.t.k.* protein in the fruit.

We have not directly measured the amount of *B.t.k.* protein produced by these transgenic plants. However, it is possible to estimate the amount of protein produced by measuring the amount of leaf material consumed by the *M. sexta* larvae before they are killed. Based on the lethality of *E. coli* cultures expressing known amounts of the truncated protein encoded by the deletion variant in pMON9711 (data not shown), we estimate that the transgenic plants are producing on the order of 50 ng of *B.t.k.* protein per gram of leaf tissue (fresh weight). It should be possible to increase the level of protein production substantially through further manipulation of the chimeric genes, and we are currently investigating this possibility. We have seen differences in the level of expression of the *B.t.k.* gene from plant to plant as measured by the bioassay. These differences are consistent with the plant to plant variation seen with other chimeric genes in transgenic plants[21,28].

This report demonstrates the feasibility of genetically engineering insect tolerance into transgenic crop plants

through the expression of a *B.t.k.* insect control protein gene. Plants producing their own insecticidal proteins increase the selectivity of control since only pests that attack the plant will be affected. These plants also afford continuous control in contrast to the sporadic control provided by present application technology. Genetically engineered insect tolerant crop plants should prove to be a valuable addition to the strategies currently available for insect pest control.

EXPERIMENTAL PROTOCOL

DNA manipulations. Recombinant DNA techniques were as described by Maniatis et al.[29] DNA sequence analysis of the *B.t.k.* gene was performed by the dideoxy method of Sanger et al.[30] utilizing 80 cm buffer gradient gels[31] following subcloning of individual restriction fragments into M13 vectors mp8 and mp9[32].

Construction of deletion variants of the *B.t.k.* gene. pMAP9, pMAP11 and pMON9906 are simple deletions of restriction fragments from the *B.t.k.* gene in pMAP4. For construction of pMAP9, pMAP11 and pMON9906, pMAP4 was digested with NruI plus ScaI, HindIII and XbaI, respectively. Following digestion, the DNA was recircularized by treatment with T4 DNA ligase and transformed into *E. coli*. Appropriate clones containing the desired deletion variants were identified by restriction enzyme analysis. pMON9905 was constructed by digestion of pMAP4 with KpnI. Following digestion, the DNA was ligated with a synthetic oligonucleotide pair of sequence:

5′-CTAGTAAA-3′
3′-CATGGATCATTTCTAG-5′.

This synthetic linker contains a KpnI overhang and two termination codons in frame with the *B.t.k.* protein coding sequence at the KpnI site at nucleotide 2174. In addition, when ligated to itself, the linker creates a BglII site. Following transformation of *E. coli*, a clone containing the desired deletion and a BglII site was identified by restriction enzyme analysis and designated pMON9905. pMON5323 was constructed in similar fashion except that the *B.t.k.* gene was first digested with BclI. After digestion the single-stranded ends were made flush by treatment with DNA polymerase I Klenow fragment. The DNA was then ligated with a synthetic oligonucleotide pair of sequence:

5′-TAGTAGGTAGCTAGCCA-3′
3′-ATCATCCATCGATCGGTCTAG-5′.

This synthetic linker contains termination codons in all three reading frames. In addition, when ligated to itself, the linker creates a BglII site. When ligated to pMAP4 DNA treated as described above, this linker creates a gene which encodes 646 amino acids of the *B.t.k.* protein plus three amino acids encoded by the linker. Following transformation of *E. coli*, a clone containing the truncated gene and a BglII site was identified by restriction enzyme analysis and designated pMON5323.

Construction of plant transformation vectors containing truncated *B.t.k.* genes. An NcoI site was engineered at the ATG initiation codon of pMAP4 by site-specific[33] mutagenesis to create plasmid pMAP17. The 5′ end of the gene was further modified by addition of a synthetic oligonucleotide of sequence: 5′-GGATCCAGATCTGTTGTAAGGAGTCTAGA-3′ just 5′ to the NcoI site to create plasmid pMON294. This linker contains a BglII site (underlined). To construct a plasmid vector for plant transformation containing a *B.t.k.* gene truncated at the KpnI site at nucleotide 2174, pMON294 was digested with KpnI, ligated with the synthetic linker utilized in the construction of pMON9905 described above and then digested with BglII. A 2.2 kb BglII fragment containing the truncated gene was isolated and ligated with BglII digested pMON316[21]. A clone in which the 5′ end of the *B.t.k.* gene was adjacent to the CaMV 35S promoter was isolated and designated pMON9711. To construct a plasmid containing a *B.t.k.* gene truncated at the BclI site at nucleotide 1935, pMON294 was digested with BclI, treated with DNA polymerase I Klenow fragment, ligated with the synthetic linker utilized in the construction of pMON5323 described above and then digested with BglII. A BglII fragment of approximately 2.0 kb containing the truncated gene was ligated with BglII digested pMON316. A clone in which the 5′ end of the *B.t.k.* gene was adjacent to the CaMV 35S promoter was isolated and designated pMON9713.

***Agrobacterium*-mediated transformation and regeneration of transgenic plants.** Plant transformation vectors pMON9711 and pMON9713 were introduced into *Agrobacterium tumefaciens* strains containing disarmed Ti plasmids as described by Fraley et al.[22] The *Agrobacterium* strains containing these vectors were used to transform explants of tomato *(Lycopersicon esculentum)* line VF36, and transgenic tomato plants were recovered as described by McCormick et al.[23] Primary transgenic plants regenerated from tissue culture were designated R0. The R0 plants were selfed, and first generation progeny plants were designated R1. R1 plants were assayed for inheritance of the transforming DNA by scoring for nopaline synthesis and for kanamycin resistance. R1 plants, which had inherited the transforming DNA, were selfed and the second generation plants were designated R2. Putative homozygous R1 plants were identified as those that did not segregate any nopaline negative, kanamycin sensitive progeny in the R2 generation.

RNA filter hybridization. Total RNA was isolated from tomato tissue[34], and poly A^+ RNA was purified by oligo-dT cellulose chromatography. Poly A^+ RNA was fractionated on agarose gels containing formaldehyde[29] and blotted directly onto GeneScreen membranes in 10× SSC buffer. Blots were hybridized with a ^{32}P-labeled BamHI-PstI restriction fragment from pMAP17, which contains the entire *B.t.k.* gene. Hybridization was at 42 °C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's reagent, 1% SDS and 100 μg/ml salmon sperm DNA. The final posthybridization wash was at 50 °C in 0.1× SSC, 0.2% SDS.

Insect bioassays. *E. coli* cultures expressing the *B.t.k.* gene were assayed for lethality to neonate *Manduca sexta* (tobacco hornworm) larvae by incorporation into an artificial diet as previously described[17]. Tomato plants containing the *B.t.k.* gene were tested for lethality to *M. sexta* using both an isolated leaf feeding assay and a whole plant feeding assay. In the isolated leaf assay, one or more tomato leaves from a single plant were excised and placed in a Petri dish on water saturated filter paper. Ten neonate larvae were applied to the leaves and allowed to feed for four days at which time they were scored for mortality and weight gain. For the whole plant assay, ten neonate larvae were applied to the leaves of tomato plants (typically four to eight weeks after planting) and allowed to feed for four to seven days. At the end of the assay the larvae were scored for mortality and weight gain, and the plants were rated for amount of damage. Transgenic tomato plants were also assayed for lethality to *Heliothis virescens* (tobacco budworm) larvae and to *Heliothis zea* (corn earworm) larvae in a modified isolated leaf feeding assay. Individual leaves were placed in Petri dishes, and only a single three to four day old larva (2nd or 3rd instar) was added to each dish. After four days the larvae were scored for mortality and weight gain. A total of ten to twenty *Heliothis* larvae were tested on isolated leaves from each plant. All insect bioassays were performed in duplicate or triplicate. Corrected mortality was calculated according to Abbott's formula[35].

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